REMARKS

THE CLAIM AMENDMENTS

Claim 1 has been amended to direct the claim to the detection of a nucleic acid analyte selected from the group consisting of DNA, human sequences of interest, endogenous genes, and segments thereof. Support for this change is found throughout the specification (*see, e.g.*, page 5 line 14, page 8, line 11, and page 17, lines 1-5) and also in pre-amendment claim 3. With the amendment to claim 1, claim 3 has been amended to delete any redundant subject matter.

Claims 1, 27, and 28 have also been amended to reintroduce the "optionally" to method step (a)(iii), which was removed in the Amendment filed on June 28, 2004.

No new matter has been added to the application with claim amendments set forth herein.

THE LEGAL STANDARD FOR THE *PRIMA FACIE* CASE

The *prima facie* case is a procedural tool which, as used in patent examination, means not only that the evidence of the prior art would reasonably allow the conclusion the Examiner seeks, but also that the prior art compels such a conclusion if the applicant produces no evidence or argument to rebut it. *In re Spada,* 911 F.2d 705 (Fed. Cir. 1990). If examination at the initial stage does not produce a *prima facie* case of unpatentability, then without more, the applicant is entitled to a grant of the patent. *In re Oetiker,* 977 F.2d 1443 (Fed. Cir. 1992).

The Examiner's *prima facie* case is based on the obviousness of the claimed invention over the cited references.

To establish a *prima facie* case of obviousness, two basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teachings. Second, there must be a reasonable likelihood of success in view of the prior art. *Brown V. Williamson Tobacco Corp. v. Phillip Morris, Inc.*, 229 F.3d 1120 (Fed. Cir. 2000). Furthermore, the teachings or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991). An obviousness analysis that relies upon the applicant's disclosure rather than the prior art reference is improper as being based upon an impermissible hindsight reconstruction. *In re Deuel*, 51 F.3d 1551, 1558 (Fed. Cir. 1995).

The following discussion outlines the Examiner's *prima facie* case of obviousness and provides arguments in response to same.

THE REJECTION OVER ANTAO ET AL. IN VIEW OF XU ET AL.

Claims 1, 3-4, 6-23, and 27-33 stand rejected under 35 U.S.C. § 103(a) as obvious over Antao et al. in view of Xu et al. This rejection is respectfully traversed.

As recited in independent claim 1, the present invention relates to a method for *in situ* detection of a nucleic acid analyte within a sample of biological material using bDNA hybridization...wherein the nucleic acid analyte is selected from the group consisting of DNA, human sequences of interest, endogenous genes, and segments thereof.

As recited in independent claim 27, the present invention relates to a method for identifying the position of a nucleic acid analyte within a sample of biological material using bDNA hybridization...

As recited in independent claim 28, the present invention relates to a method for detecting a nucleic acid analyte within a sample of biological material comprising performing bDNA hybridization to detect the nucleic acid analyte *in situ*, wherein the method has a sensitivity sufficient to detect from about 1 to about 10 copies of the nucleic acid analyte in the biological material...

As explained by Inventor Daryn Kenny at paragraph 6 of the attached Declaration filed pursuant to 37 C.F.R. § 1.132, the present invention is unique over the art available at the time of the invention because the present invention allows for the detection of very small quantities of DNA *in situ* using bDNA hybridization for signal amplification. Prior to the invention, the bDNA assay was only capable of detecting RNA *in situ*. Because expression levels of RNA are significantly greater than that of DNA, *in situ* RNA assays do not require the sensitivity that *in situ* DNA assays require. An inherent problem encountered in all *in situ* DNA assays is background noise. Because RNA has significantly higher expression levels than DNA, the RNA signal is capable of being seen over background noise. The reason for the high background noise in *in situ* bDNA assays is that large quantities of oligonucleotides are required in order to perform the bDNA assay. The present invention is aimed specifically at overcoming these obstacles inherent in the application of *in situ* bDNA assays in order to test for DNA in whole tissue samples, to detect the subcellular localization of nucleic acid analytes within a sample of biological material, and to detect 1-10 copies of a nucleic acid analyte in biological material.

Antao et al. describes an *in situ* RNA assay using bDNA; in particular, for the assays described therein, Antao et al. uses two RNA targets: HIV-1 viral RNA and hnRNP A2/B1 mRNA (page 87). As is evident from the figures on pages 88, 90, and 91 of Antao et al., background in the RNA experiments is significant. Although Antao et al. suggests that the *in situ* bDNA technology is in the process of testing for the detection of DNA in tissues (*see*, page 90, 3rd full para.), there is no published evidence to support the suggestion. While Antao et al. references a manuscript in preparation, a Medline search performed using the terms "author, Grogan, T" and "in situ" does not find any references describing the use of *in situ*

bDNA assays for the testing of DNA samples; accordingly, it follows that the referenced manuscript was never published. Without any showing that the *in situ* bDNA assay of Antao et al. has success with DNA samples, the ordinary artisan would not be led to the claimed invention because the *in situ* bDNA assay of Antao et al. would not provide the highly sensitive results of the claimed invention.

The Examiner cites Xu et al. for the washing step, which the Examiner notes is not disclosed in Antao et al. (Office Action, page 4). The washing step of Xu et al., however, does not correct the deficiencies of Antao et al. Xu et al. does not teach a bDNA assay, it teaches the use of hapten-labeled probes to screen for mRNA *in situ*; accordingly, it follows that both Antao et al. and Xu et al. only provide information relating to the screening of RNA and do not teach or suggest the use of the assays for the screening of DNA (*see*, Antao et al. page 87 under "Results and Discussion" and Xu et al., page 99 under "General Strategy").

As explained by Inventor Daryn Kenny at paragraph 8, at the time of the invention, the present inventors applied the reaction conditions described in Antao et al. and Xu et al. to a bDNA *in situ* assay to screen for DNA signals and the results were not satisfactory. The background noise in the reactions was such that DNA signals could not be seen. Because of the inability of the Antao et al. and Xu et al. assays to provide adequate use of a bDNA *in situ* assay for the detection of DNA, the present inventors undertook to correct the deficiencies in the prior art – the result being the invention described in the instant application.

With the changes made by the present invention, the inventors made the surprising and unexpected discovery that the subcellular location of a nucleic acid analyte and as few as one to ten copies of a nucleic acid analyte could be identified using the claimed method (claims 27 and 28 et seq., respectively). On this point, applicants note that the Examiner did not provide any arguments in support of the obviousness of claims 27 and 28 et seq. over Antao et al. in view of Xu et al. Indeed, there is nothing in Antao et al. or Xu et al. that would lead the ordinary artisan to modify the teachings of Antao et al. or Xu et al. such that the method of Antao et al. in view of Xu et al. could be used to detect the subcellular localization of a nucleic acid analyte (claim 27) or to increase the sensitivity of the assays disclosed in Antao et al. in view of Xu et al. such that one to ten copies of a nucleic acid analyte maybe detected (claims 28 et seq.). Because Antao et al. in view of Xu et al. provide no motivation for the ordinary artisan to modify the assays as disclosed in Antao et al. in view of Xu et al. to detect DNA *in situ* using bDNA or to detect the subcellular localization of an analyte within a biological sample or to detect one to ten copies of a nucleic acid analyte within a sample, the ordinary artisan could not have a reasonable expectation of successfully arriving at the claimed invention merely by combining the teachings of Antao et al in view of Xu et al.

Because the invention as recited in independent claim 1 et seq., independent claim 27, and independent claim 28 et seq. is not rendered obvious by the hypothetical combination of Antao et al. in view of Xu et al., applicants respectfully request reconsideration and withdrawal of this rejection.

THE REJECTION OVER SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., AND XU ET AL.

Claims 1, 3-4, 6-13, 16, 17, and 20-27 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. This rejection is respectfully traversed.

Independent claims 1 and 27 are described above in the discussion of the rejection of the claimed invention over Antao et al. in view of Xu et al.

Schaeren-Wiemers et al. describes a non-radioactive *in situ* hybridization assay with digoxigenin ("DIG")-labeled cRNA probes for localization of selected mRNA species in tissue sections and cultured cells from the central nervous system. As acknowledged by the Examiner, Schaeren-Wiemers et al. does ***not*** teach or suggest using bDNA for detecting the mRNAs disclosed therein. Thus, like Antao et al. and Xu et al., the experiments of Schaeren-Wiemers only apply to the detection of *in situ* RNA. Further separating Schaeren-Wiemers et al. from the claimed invention is the evident fact that Schaeren-Wiemers et al. does not apply the bDNA assay to amplify the signal of the RNA and relies instead upon DIG-labeled cRNA probes.

Cao et al. does not correct the deficiencies of Schaeren-Wiemers et al. because as mentioned previously, the bDNA *in situ* assay described in Cao et al. only applies to the screening of RNA, not DNA.

Nolte does not correct the deficiencies of Schaeren-Wiemers et al. in view of Cao et al. Nolte is a review paper that describes bDNA signal amplification to detect mRNAs in clinical samples. At page 231, Nolte suggest that the increased sensitivity of bDNA may cast new applications for the technology in filter and *in situ* hybridization assays; however, Nolte does not provide any guidance on how this can be achieved. *See*, Paragraph 10 of the Declaration of Inventor Daryn Kenny, Ph.D. Because the Declaration of Daryn Kenny, Ph.D. provides evidence that the conditions for bDNA *in situ* assays for the detection of RNA cannot be imported successfully to the detection of DNA, it follows that the suggestion made in Nolte, without more, cannot serve to render the claimed invention obvious. *See*, Paragraph 8 of the Declaration of Inventor Daryn Kenny, Ph.D.

Decimo et al. does not correct the deficiencies of Antao et al. in view of Schaeren-Wiemers et al. Cao et al., and Nolte. Decimo et al. describes *in situ* hybridization to screen for RNAs. Decimo et al.

does not teach or suggest that the RNA may be detected using bDNA; rather, the RNA is detected in Decimo et al. with radiolabeling (*see*, pages 186-188 and 196-197). The Examiner cites Decimo et al. for the basic conditions for conducting *in situ* hybridization assays. These steps fail to correct the cumulative failings of all of the prior references, that is, that the bDNA assay is not taught for the detection of *in situ* DNA.

Xu et al. does not correct the deficiencies of Schaeren-Wiemers et al. in view of Cao et al., Nolte, and Decimo et al. Xu et al. teaches *in situ* hybridization of mRNA with hapten-labeled probes, such as digoxigenin- ("DIG"), fluorescein-, and biotin-UTP that can be used to make labeled RNA probes. Xu et al. does not teach or suggest that bDNA may be used for signal amplification. Although Xu et al. uses a completely different signal detection system from the bDNA signal amplification system of the claimed invention, the Examiner attempts to compare the amounts of the two differing detection probes. Paragraph 11 of Inventor Daryn Kenny's Declaration explains why it is unreasonable to substitute the concentrations of DIG-labeled cRNA probe with the oligonucleotides required for bDNA.

Turning to the specifics of the Examiner's argument, at pages 10-11 of the Office Action, the Examiner asserts that Xu teaches average *in situ* probes between 32 to 36 nucleotides in length. Applicants have reviewed Xu et al. in detail and have not found any teaching therein that the *in situ* probes are 32-36 nucleotides in length; rather, applicants have found teachings in Xu et al. that the DIG-labeled probes are quite large. At page 89 of Xu et al., it is provided that longer probes give stronger signals, so it is best to synthesize as long a probe as possible, which should then be size-reduced as described in Protocol 1 (pages 91-92) if it is greater than 1 kb in length. Protocol 1 provides a procedure to reduce the length of a probe from 1 kb or greater to approximately 500 bases (page 92, no. 5). At page 90 of Xu et al. it is reiterated that the signal strength of the *in situ* assay disclosed therein is related to the sequence complexity of the probes, so whenever possible, long probes of approximately 0.5 to 0.3 kb should be used; however, since long probes penetrate less efficiently into the tissue to obtain optimal signals, the RNA probe is reduced in size to an average of approximately 500 pb. If applicants have overlooked any teaching in Xu et al. relating to probes that are 32 to 34 nucleotides in length, applicants respectfully request that the Examiner direct applicants' attention to such teaching.

Since applicants have been unable to find the relevant teaching in Xu et al., applicants must also traverse the Examiner's assertion that Xu et al. teaches approximately 0.91 pmoles of target probe. Applicants have carefully reviewed the Examiner's calculation and cannot discern from where the Examiner derived the information relating to the average molecular weight of each base as the Examiner did not cite any support for the 130 molecule weight of the base. It is applicants' understanding that the average molecular weight for a base pair is 660 pg/pmol and thus, it would follow that a single nucleotide

is 330 pg/mol. Further, contrary to the Examiner's assertion that Schaeren-Wiemers et al. used 200 µL of hybridization solution, at column 2 of page 433 of Schaeren-Wiemers et al., it is disclosed that 200 µL of prehybridization solution is used, but only 50-100 µL of hybridization solution is used for hybridizing the tissue sections.

As is evident from the discussion set forth herein, none of the cited references, save Cao et al., teach or suggest the use of bDNA for signal amplification of *in situ* hybridization assays. Because Cao et al. only teaches the use of bDNA assays for the detection of RNA, ***not*** DNA, any combination of the cited references will not lead the ordinary artisan to the successful detection of DNA *in situ* using bDNA for signal amplification. As previously noted, the mere one-line suggestion in the last sentence of the last paragraph of Nolte provides absolutely no guidance for the ordinary artisan to modify the bDNA RNA *in* situ assay so that it is capable of screening for DNA. Indeed, Nolte provides no statement that would lead the ordinary artisan to conclude that the lack of sensitivity required for the high expression RNA assays would lead to an inability to detect the weaker DNA signal in the DNA assays.

With respect to the Examiner's rejection of claim 27, applicants note that the Examiner has not cited ***any*** art that supports the position that the mere detection of a signal indicates the position of a nucleic acid analyte within a cell (*see*, Office Action, page 11). Both the Federal Circuit and the Office, the latter by way of the MPEP, have put prohibitions upon Examiner's taking of notice without documentary support.

Where an Examiner chooses to take notice of facts beyond the record for the *prima facie* case, those facts must be "capable of such instant and unquestionable demonstration as to defy dispute." *In re Alhert*, 24 F.2d 1099, 1091 (CCPA 1970). It is ***not*** appropriate for an Examiner to take official notice of facts without citing a prior art reference where the facts asserted to be well-known are not capable of *instant and unquestionable demonstration as being well-known. Id.* For example, assertions of technical facts in esoteric technology or specific knowledge of the prior art must always be supported by citation to some reference work recognized as standard in the pertinent art. *Id.*; *see also*, MPEP § 2144.03, 8th ed., Aug. 2001, Rev. Feb. 2003, pp. 2100-131-2100-132; *In re Grose*, 592 F.2d 1161, 1167-1168 (CCPA 1979) ("[W]hen the PTO seeks to rely upon a chemical theory, in establishing a prima facie case of obviousness, it must provide evidentiary support for the existence and meaning of the theory."). More importantly, it has also been established that an Examiner is not at liberty to assert that the state of the art is common knowledge; the state of the art must ***always*** be shown by way of documentary evidence. *See*, MPEP § 2144.03, p. 2100-132; *In re Eynde*, 480 F.2d 1364, 1370 (CCPA 1973) ("[W]e reject the notion that judicial or administrative notice may be taken of the state of the art. The facts constituting the state of the art are normally subject to the possibility of rational disagreement among reasonable men and are

not amenable to the taking of such notice."). All of these well-established principles of administrative notice were reiterated by the Federal Circuit in the important case, *In re Zurko*, 258 F.3d 1379, 1385 (Fed. Cir. 2001) ("[T]he Board cannot simply reach conclusions based on its own understanding or experience – or on its assessment of what would be basic knowledge or common sense.").

In light of the foregoing, applicants respectfully request that the Examiner provide documentary evidence attesting to the basis of the knowledge asserted against claim 27 in this rejection.

Notwithstanding the foregoing, directing the Examiner's attention to paragraph 12 of the attached Declaration of Inventor Daryn Kenny, Ph.D., there, Dr. Kenny explains why the Examiner's position that the subject matter of the claim 27 is evident from the mere detection of the nucleic acid within the cell is ***not*** an accurate statement. The detection of the precise location of a nucleic acid analyte within a cell, be it RNA or DNA or a fragment thereof, is not readily apparent from the mere detection of a signal. Because autoradiography and chemiluminescence produces signals that radiate outwards, identifying the position of a nucleic acid analyte within a cell is not possible using these traditional means of signal detection. Through the use of bDNA under the precise conditions set forth in the instant application, the present inventors were able to refine signal amplification such that the background noise created from traditional radiolabelling and chemiluminescence is no longer a hindrance to the identification of the precise location of a nucleic acid analyte within a cell.

Because the present invention is not rendered obvious by the hypothetical combination of Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al., applicants respectfully request reconsideration and withdrawal of this rejection.

THE REJECTION OVER SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., XU ET AL., AND SARTO ET AL.

Claim 5 stands rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. as applied to claims 1, 3-4, 6-13, 16, 17, and 20-27 and further in view of Sarto et al. (U.S. Patent No. 6,022,689). This rejection is respectfully traversed.

Claim 5 is dependent upon claim 1. Because claim 1 is not obvious over the hypothetical combination of Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al., it follows that the additional teachings of Sarto et al. cannot serve to render claim 5 obvious.

Because claim 5 is not rendered obvious by Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al, and Sarto et al., applicants respectfully request reconsideration and withdrawal of this rejection.

THE REJECTION OVER SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., XU ET AL., AND KERN ET AL.

Claims 14 and 15 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. as applied to claims 1, 3-4, 6-13, 16, 17, and 20-27 and further in view of Kern et al. This rejection is respectfully traversed.

Claims 14 and 15 are dependent upon claim 1. Because claim 1 is not obvious over the hypothetical combination of Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al., it follows that the additional teachings of Kern et al. cannot serve to render claims 14 and 15 obvious.

Because claims 14 and 15 are not rendered obvious by Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al, and Kern et al., applicants respectfully request reconsideration and withdrawal of this rejection.

THE REJECTION OVER SCHAEREN-WIEMERS ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., XU ET AL., AND SIADAT-PAJOUH ET AL.

Claims 18 and 19 stand rejected under 35 U.S.C. § 103(a) as obvious over Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. as applied to claims 1, 3-4, 6-13, 16, 17, and 20-27 and further in view of Siadat-Pajouh et al. This rejection is respectfully traversed.

Claims 18 and 19 are dependent upon claim 1. Because claim 1 is not obvious over the hypothetical combination of Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al., it follows that the additional teachings of Siadat-Pajouh et al. cannot serve to render claims 18 and 19 obvious.

Because claims 18 and 19 are not rendered obvious by Schaeren-Wiemers et al. in view of Cao et al., Nolte, Decimo et al., Xu et al, and Siadat-Pajouh et al., applicants respectfully request reconsideration and withdrawal of this rejection.

THE REJECTION OVER SIADAT-PAJOUH ET AL. IN VIEW OF CAO ET AL., NOLTE, DECIMO ET AL., AND XU ET AL.

Claims 28-35 stand rejected under 35 U.S.C. § 103(a) as obvious over Siadat-Pajouh et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al. This rejection is respectfully traversed.

Independent claim 28 is described above in the discussion of the rejection of the claimed invention over Antao et al. in view of Xu et al.

Siadat-Pajouh et al. describes the use of *in situ* hybridization to detect one to five copies of the human papillomavirus ("HPV") genome in SiHa cells using DIG tail-labeled oligonucleotides. In

performing the *in situ* hybridization assay for HPV, Siadat-Pajouh et al. undertook five different fluorescence-based *in situ* hybridizations ("FISHs"). Of the five methods, only one, Method 1, was able to detect an HPV-16 DNA in the SiHa cells. The Examiner acknowledges that Siadat-Pajouh et al. does ***not*** teach using bDNA for signal amplification (Office Action, page 16).

The Examiner cites Cao et al. and Nolte for the teachings of bDNA and Decimo et al. and Xu et al. for different hybridization conditions.

The experiments of Siadat-Pajouh et al. demonstrate the inherent difficulties in obtaining a reproducible signal for *in situ* DNA. As noted unequivocally in Siadat-Pajouh et al., only the DIG tail-labeled oligonucleotides of Method 1 were able to produce a signal in the FISH assay, while DIG-labeled DNA probes (Method 2), fluorescein-15-d-ATP-labeled oligonucleotides (Method 3), fluorescein-15-d-ATP labeled DNA probes (Method 4), and biotin-DNA-labeled probes (Method 5) were not capable of detecting reproducible DNA signals. Despite the disclosure of the inherent difficulty in obtaining a signal of *in situ* DNA, the Examiner draws the conclusion that substitution of bDNA for the DIG tail-labeled oligonucleotides of Method 1 will result in a reproducible signal (Office Action, page 22). Applicants submit that it is impossible to come to this conclusion through the combined teachings of the cited references.

As previously noted, each of the cited secondary references relate to conditions required for the detection of *in situ* RNA, ***not*** DNA. As clearly indicated in Siadat-Pajouh et al., the reproducibility of *in situ* DNA assays is extremely difficult to obtain; thus, the Examiner's assertion that the ordinary artisan would have a reasonable expectation of success by substituting the bDNA and hybridization conditions of the secondary references into the assay of Siadat-Pajouh et al. because Siadat-Pajouh et al. teaches that "signal amplifications may be performed in various ways" (Office Action, page 22) is ***not*** an accurate statement. Siadat-Pajouh et al. presents five different labeling methods and ***only one works***. Thus, while Siadat-Pajouh et al. may provide five techniques for signal amplifications, Siadat-Pajouh et al. also teaches that at the most, only one in five techniques may have any success in the detection of HPV DNA.

Because Siadat-Pajouh et al. clearly demonstrates the difficulty in obtaining results for 1-5 copies of HPV-DNA using the DIG tail-labeled oligonucleotides disclosed therein and the secondary references all relate to the screening of the much more easily detectable RNA, it follows that the ordinary artisan would not have a reasonable expectation of succeeding in substituting the techniques of Cao et al., Nolte, Decimo et al., and Xu et al., without the benefit of the teachings in the instant application. *See*, *In re Deuel*, *supra* (An obviousness analysis that relies upon the applicant's disclosure rather than the prior art reference is improper as being based upon an impermissible hindsight reconstruction) and the Declaration of Inventor Daryn Kenny, Ph.D., paragraphs 14 and 15. In light of the foregoing, applicants submit that

the Examiner has failed to establish a *prima facie* case of obviousness of claims 28-33 over Siadat-Pajouh et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al.

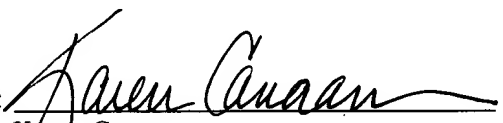
Because the present invention is not rendered obvious by the hypothetical combination of Siadat-Pajouh et al. in view of Cao et al., Nolte, Decimo et al., and Xu et al., applicants respectfully request reconsideration and withdrawal of this rejection.

CONCLUSION:

The foregoing discussion addresses and provides arguments in rebuttal of each of the Examiner's rejections. Because the claimed invention is not rendered obvious over the cited art, it follows that the applicants are entitled to a patent grant on the claimed invention. *See*, *In re Spada*, and *In re Oetiker*, *supra*. Because the application will be in condition for allowance upon entry of this amendment, applicants respectfully request reversal of all claim rejections and passage of this application to issue.

Should the Examiner have any questions concerning this response, she is welcome to contact the undersigned attorney at 650-330-4913 or at canaan@reedpatent.com.

Respectfully submitted,

By: 
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ARTICLE

Detection of Viral Infection and Gene Expression in Clinical Tissue Specimens Using Branched DNA (bDNA) In Situ Hybridization

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SUMMARY In situ hybridization (ISH) methods for detection of nucleic acid sequences have proved especially powerful for revealing genetic markers and gene expression in a morphological context. Although target and signal amplification technologies have enabled researchers to detect relatively low-abundance molecules in cell extracts, the sensitive detection of nucleic acid sequences in tissue specimens has proved more challenging. We recently reported the development of a branched DNA (bDNA) ISH method for detection of DNA and mRNA in whole cells. Based on bDNA signal amplification technology, bDNA ISH is highly sensitive and can detect one or two copies of DNA per cell. In this study we evaluated bDNA ISH for detection of nucleic acid sequences in tissue specimens. Using normal and human papillomavirus (HPV)-infected cervical biopsy specimens, we explored the cell type-specific distribution of HPV DNA and mRNA by bDNA ISH. We found that bDNA ISH allowed rapid, sensitive detection of nucleic acids with high specificity while preserving tissue morphology. As an adjunct to conventional histopathology, bDNA ISH may improve diagnostic accuracy and prognosis for viral and neoplastic diseases.
(J Histochem Cytochem 50:1219–1227, 2002)

KEY WORDS
branched DNA (bDNA)
cervical intraepithelial neoplasia (CIN) biopsies
human papillomavirus (HPV)
in situ hybridization

CONVENTIONAL HISTOPATHOLOGY is traditionally the first line of investigation for understanding disease. Because certain pathomorphological features are often associated with viral and neoplastic diseases, histological examination of tissues can identify diagnostic criteria for disease, determine the extent of disease progression, and provide insight for prognosis. For example, hepatic inflammation in liver biopsies distinguishes chronic hepatitis C from autoimmune chronic hepatitis (Bach et al. 1992), lymphoid tissue architecture is severely affected during human immunodeficiency virus type 1 (HIV-1) infection (Knowles and Chadburn 1992), changes in cell morphology are associated with human papillomavirus (HPV) viral infection (Arends et al. 1998), and changes in nuclear and cell morphology can indicate cancer type and stage of progression (Ishak et al. 1998). Although histopathological findings provide valuable insight into disease, an important potential adjunct is the detection of molecular markers. The complementary contributions of histopathology and molecular markers are illustrated by the early detection of cancer of the cervix and breast. For cervical cancer, detection of high-risk HPV genotypes improves the sensitivity of Pap tests identifying patients with pre-cancerous lesions (Wagner et al. 1984; Manos et al. 1999). Similarly, determination of human epidermal growth factor receptor 2 (HER2/neu) gene copy number in breast cancer biopsies aids physicians and patients in therapy selection (Ross and Fletcher 1999). Hence, the detection of molecular markers can improve the diagnostic accuracy and prognostic value of morphology-based and immunohistochemistry-based methods.

Among the methods used for detection of molecular markers, in situ hybridization (ISH) offers the unique advantage of visualizing and even quantifying clinically relevant molecules in a morphological context (Unger 2000). The application of nucleic acid target and signal amplification technologies to ISH has enabled re-

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searchers to detect as few as one or two copies of specific DNA molecules in cell preparations (Femino et al. 1998; Player et al. 2001). However, the sensitive detection of nucleic acid sequences in tissue biopsy specimens has proven more challenging. In addition to the general problems of ISH, such as diffusion of signal, fluorescence quenching, and preservation of cell morphology, effective detection of nucleic acid sequences in biopsy specimens must overcome challenges unique to tissue sections, including more limited accessibility to target sequences, increased background from nonspecific hybridization, endogenous reporter activity, and preservation of complex tissue architecture.

We recently reported the development of a branched DNA (bDNA) ISH method for detection of DNA and mRNA in whole cells (Player et al. 2001). The bDNA ISH method is a signal amplification system in which target nucleic acid sequences are hybridized to a series of synthetic oligonucleotide probes and visualized through generation of chromogenic or fluorescent signals in an alkaline phosphatase (AP)-catalyzed reaction. The bDNA ISH method is highly sensitive and can detect one or two copies of DNA per cell. Moreover, the bDNA ISH method is specific and provides precise localization, yielding positive signals that are retained within the subcellular compartment in which the target nucleic acid is localized. Our earlier work showed that bDNA signal amplification overcomes many of the challenges of ISH when applied to cell preparations. Damage to cell morphology caused by heat exposure is minimized because bDNA ISH does not require repeated cycling through elevated temperatures. Background caused by endogenous biotin is avoided because bDNA ISH does not utilize an avidin–biotin reporter system. Diffusion of the signal is not a problem because reporter probes for bDNA ISH are physically linked to the spatially fixed nucleic acid targets. Whereas our earlier studies were performed on cervical cancer cell lines, we wished to test whether bDNA ISH would provide the same sensitivity, specificity, and precise localization of nucleic acid sequences in tissue sections.

In this study we evaluated the bDNA ISH method for detection of nucleic acid sequences in clinical tissue specimens. Using normal and HPV-infected cervical biopsy specimens as a model system, we explored the cell type-specific distribution of HPV DNA and mRNA by bDNA ISH with oligonucleotide probes designed for HPV-16 and HPV-18 E6/E7 gene sequences. We assessed the distribution of HPV DNA and mRNA among the various cell types present in cervical intraepithelial neoplasias (CIN), explored the cell type-specificity of HPV localization by comparing epithelial regions with and without dysplasia, and demonstrated the specificity of bDNA ISH by comparing HPV detection in sections from HPV-16- and HPV-18-infected CIN II and normal cervical biopsies.

Materials and Methods

Specimens

Formalin-fixed, paraffin-embedded uterine cervix Leep cone biopsy specimens were obtained from Clinomics Laboratories (Frederick, MD) with Internal Review Board approval. Clinical diagnosis was provided in surgical pathology reports that specified moderate cervical dysplasia (CIN II). The catalog numbers of three HPV-positive and three normal specimens were 00B01-624, 00B01-627, 00B01-625, and H-1188-88, 00B01-631, 00B01-632, respectively. Koilocytes and dysplastic cells were identified using the Bethesda System and as described in diagnostic cytology texts (Koss 1992; Kurman and Solomon 1994).

Oligonucleotide Probes

Target-specific oligonucleotide probes for HPV-16 and HPV-18 E6/E7 and for GAPDH were designed using ProbeDesigner Software (Bayer Diagnostics; Berkeley, CA) as described previously (Bushnell et al. 1999). Other DNA oligonucleotide probes comprising the signal amplification system, including preamplifier, amplifier, and alkaline phosphatase (AP)-conjugated label probe, also have been described (Collins et al. 1997). The non-natural nucleotides 5-methyl-2′-deoxyisocytidine (isoC) and 2′-deoxyisoguanosine (isoG) were included in the binding sites of the target, preamplifier, amplifier, and AP-conjugated label probes to reduce nonspecific hybridization (Collins et al. 1997).

Pretreatment of Tissue Sections

Formalin-fixed paraffin sections of cervical tissue were dewaxed and rehydrated with standard histological methods using xylenes and graded ethanol series. For DNA detection, tissue was digested first with 100 μg/ml RNase in RNase buffer (0.5 M NaCl, 10 mM Tris, pH 9.0, 1 mM EDTA) at 37C for 1 hr and then with 12 μg/ml proteinase K in PBS (0.01 M phosphate buffer, pH 7.5) at 37C for 10 min. Immersion in Antigen Unmasking Solution (DAKO; Carpinteria, CA) for 5–40 min at 92C was substituted for proteinase K digestions in some experiments for HPV detection in basal cells and for HPV detection in tissue sections that exhibited higher background staining. After digestion, proteinase K was inactivated by postfixation in 4% paraformaldehyde in PBS at 4C for 10 min. Tissue sections were washed several times in PBS and then acetylated in 1 M triethanolamine and 0.1 M acetic anhydride. Tissue sections were then dehydrated in a graded ethanol series, denatured at 92C for 5 min in 80% formamide in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na-citrate) in a humidity chamber (Hybaid Omnislide instrument; Phoenix Research Products, Hayward, CA), followed by immersion in cold 70% ethanol and dehydration. For detection of mRNA, the procedure was the same except that there was no RNase digestion or high-temperature denaturation.

bDNA ISH

After pretreatment (described above), tissue sections were incubated with hyb 1 buffer (50% formamide, 0.2% casein, 3 × SSC, 10% dextran sulfate, 100 μg/ml salmon sperm DNA) at 40C for 30 min. HPV-specific target probes were

then added in 80 µl of fresh hyb 1 buffer at a concentration of 10 fmol/µl to each slide and coverslips were placed over slides. Hybridization was carried out in a humidified slide chamber on a slide warmer at 40C for 1–3 hr. After hybridization with target probes, tissue sections were washed in a graded series of SSC buffers from 2 × SSC to 0.1 × SSC for a total of 15 min of wash time. Coverslip-covered tissue sections were incubated with preamplifier probes at a concentration of 1 fmol/µl in hyb 2 buffer (5 × SSC, 10% dextran sulfate, 0.1% sodium dodecyl sulfate, 1 mM $ZnCl_2$, 10 mM $MgCl_2$) at 55C for 25 min, and washed in three changes of 0.1 × SSC for 5 min. Tissue sections were then incubated with amplifier probes at a concentration of 1 fmol/µl in hyb 2 buffer at 55C for 25 min and washed in three changes of 0.1 × SSC for 5 min. Finally, tissue sections were incubated with AP-conjugated label probe at a concentration of 1 fmol/µl in 80 µl hyb 2 buffer at 55C for 15 min, washed in three changes of 0.1 × SSC for 5 min, and washed once in 100 mM Tris-(hydroxymethyl)aminomethane, pH 8.0, 0.1% BRIJ-35, 1 mM $ZnCl_2$, 10 mM $MgCl_2$. Fast Red staining buffer (K0597; DAKO) containing 5 µm levamisole was prepared immediately before application to tissue sections and color was developed for 4–10 min. Nuclei were counterstained for 40 sec with Gills 1 hematoxylin (American Histology Reagent; Modesto, CA), and coverslips were placed on slides for microscopy. Digital images were captured using a Nicon Eclipse microscope, Polaroid digital camera and software, Dell computer with Microsoft Windows 2000 operating system, and combined into figures using Adobe Photoshop 5.5.

Results

Specific Detection of HPV E6/E7 mRNA in CIN II Lesions

A total of 50 serial sections of CIN II tissue from cervical Leep cone biopsy number 627 were analyzed in 12 consecutive experiments using bDNA ISH. A montage of several photographs depicting the distribution of HPV-16 E6/E7 mRNA in CIN II tissue sections is shown in Figure 1. As shown in Figure 1A, hybrid-

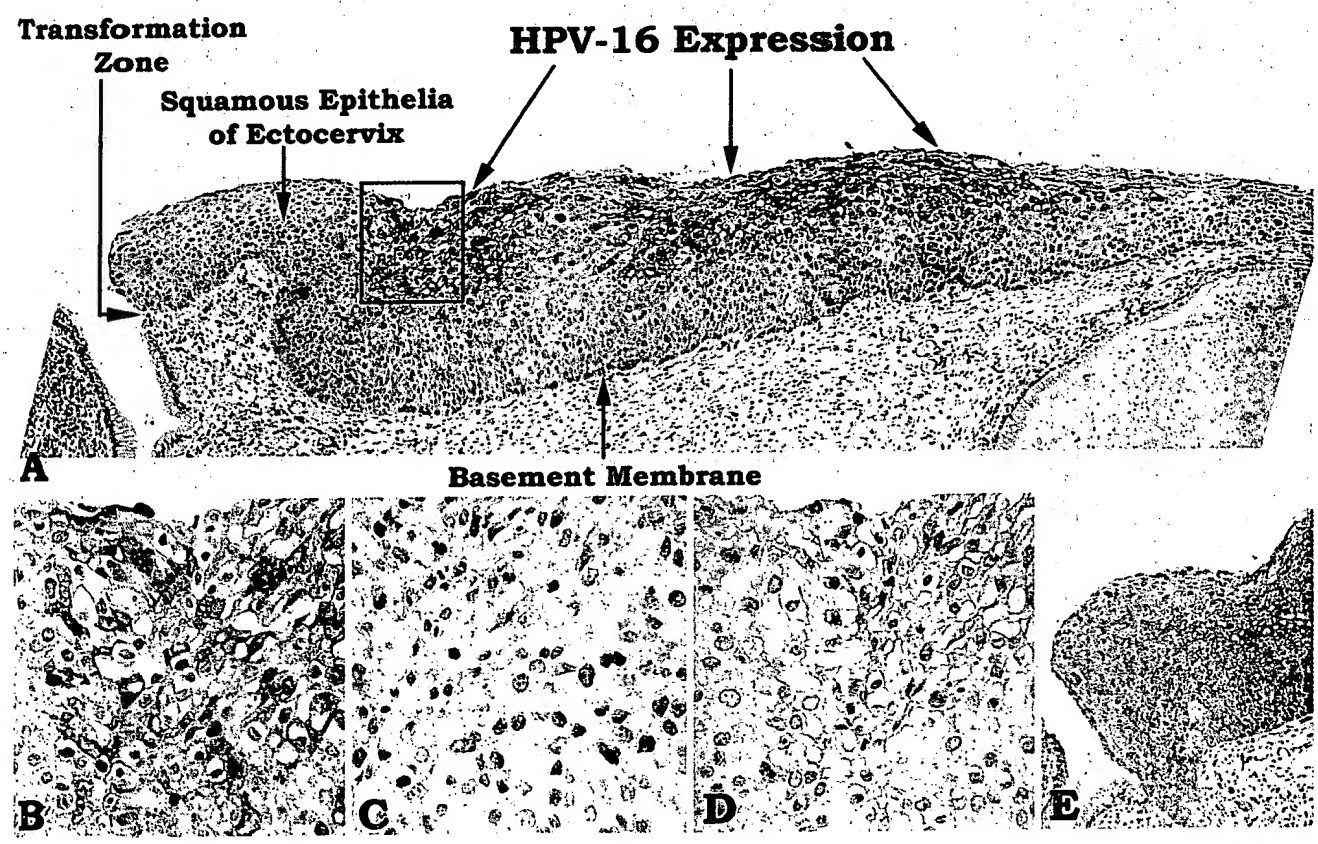

Figure 1 Localization of HPV-16 E6/E7 mRNA and DNA in sections of CIN II tissue (specimen 627) using bDNA ISH. The presence of viral sequences is visualized by deposition of the Fast Red alkaline phosphatase reaction product (red). For visualization of cell nuclei, sections were counterstained with hematoxylin (blue). **(A)** Hybridization of HPV-16 E6/E7 probes to mRNA yields signal in regions of cells with koilocytic changes but not in regions of epithelial dysplasia or in basal cells. **(B)** High-magnification view of boxed area from A shows that HPV-16 E6/E7 mRNA is predominantly localized to the cytosol in most koilocytic cells, although HPV-16 E6/E7 mRNA also is present in nuclei of some of these cells. **(C)** Hybridization of HPV-16 E6/E7 probes to DNA in an adjacent tissue section yields signal localized predominantly to nuclei of cells in areas of koilocytic changes. **(D)** Control showing that HPV-18 E6/E7 probes do not hybridize to this tissue. **(E)** Control showing that GAPDH mRNA was detected in cells throughout the squamous epithelia. Original magnifications: **A,E** ×200; **B–D** ×600.

ization with HPV-16 E6/E7 target probes in tissue sections prepared for mRNA detection consistently yielded signal in the top one third to one half of the squamous epithelia in areas of cells with koilocytic changes, indicating that HPV-16 E6/E7 mRNA was present in these cells. HPV-16 E6/E7 expression was associated with regions of cells that exhibited several of the cytopathic changes that characterize high-grade squamous intraepithelial lesions (HSIL) and/or HPV infection, including high nuclear/cytoplasmic ratios, atypical nuclei with a heterogeneous size and shape, binucleation, and perinuclear clear zones (Koss 1992; Kurman and Solomon 1994). Given the unique perinuclear halo and nuclear atypia, the HPV-16 E6/E7-expressing cells appeared to be koilocytes (Koss 1992). Located between the patches of HPV-16 E6/E7-expressing koilocytes were regions of dysplasia in which HPV-16 E6/E7 mRNA was not detected. In some sections, HPV-16 E6/E7 mRNA was detected at the transformation zone (not shown). In this and other sections of this biopsy, HPV-16 E6/E7 mRNA typically was not detected in regions of epithelial dysplasia, basal cells, proliferating squamous cell precursors, columnar epithelium of the endocervix, or underlying stromal cells.

Spurious staining of the mucus-rich columnar epithelia associated with follicles and other tissue regions was observed in some specimens more than others, regardless of HPV-16 E6/E7 mRNA detection. In most specimens this nonspecific staining was reduced significantly by the addition of acetylation and/or antigen retrieval pretreatment. Another kind of spurious staining was observed in which random deposition of the Fast Red reporter substrate occurred over a few stromal cells (right side of Figure 1A) and over a few cells at lower left of Figure 1D. This staining pattern was not reproduced on adjacent sections and was therefore clearly different from cell type- and probe-specific staining observed in koilocytes that was reproducible on adjacent sections throughout the biopsy.

The highly localized substrate deposition and low background staining of bDNA ISH allowed identification of distinct HPV-16 E6/E7-expressing cells within a tightly packed heterogeneous epithelium containing many cells without detectable HPV-16 E6/E7 mRNA. Figure 1B is a higher-magnification view of the boxed area in Figure 1A. In this region there was a mixture of stained and unstained cells, and several of the cells that stained positive for HPV-16 E6/E7 mRNA also featured cytopathic changes such as multiple nuclei or koilocytic atypia. The subcellular distribution of HPV-16 E6/E7 mRNA was predominantly localized to plasma membrane-associated cytosol and in the nuclei of a few cells but was excluded from the clear perinuclear portion of the cytoplasm. Because the AP-conjugated reporter probe was linked to the target nucleic acid, the AP staining reaction resulted in the precipitation of reporter substrate Fast Red in specific cells without diffusion into adjacent cells. Therefore, individual koilocytes that expressed HPV-16 E6/E7 mRNA were clearly distinguished from adjacent cells that were negative for HPV-16 E6/E7 mRNA.

As a control, an adjacent section prepared for mRNA detection was hybridized with GAPDH-specific target probes. As shown in Figure 1E, this yielded signal that was distributed in cells throughout the squamous epithelium uniformly between koilocytes and dysplastic regions, and also in endocervical columnar epithelium and stromal cells. Detection of GAPDH transcripts throughout the squamous epithelia is consistent with the well-known ubiquitous expression pattern of this housekeeping gene and confirms that this tissue contained RNA available for hybridization to probes.

Comparative Distribution of HPV-16 E6/E7 mRNA and DNA

Adjacent sections of CIN II tissue were analyzed to determine the relative distribution of HPV-16 E6/E7 mRNA and DNA. As shown in Figure 1C, hybridization with HPV-16 E6/E7 target probes in adjacent tissue sections prepared for DNA detection reproducibly yielded signal that was localized primarily to cell nuclei, indicating that HPV-16 E6/E7 DNA was present in regions of cells that expressed HPV-16 E6/E7 mRNA. An adjacent section hybridized with HPV-18 E6/E7 target probes yielded no signal above background (Figure 1D), indicating that the signal detected with HPV-16 E6/E7 target probes was specific. The distribution of HPV-16 E6/E7 DNA in squamous epithelia was generally more extensive than that of HPV-16 E6/E7 mRNA throughout the multiple tissue biopsies examined. For example, HPV-16 E6/E7 DNA was detected in individual basal cells and parabasal cells in addition to areas of cells with koilocytic changes. HPV-16 E6/E7 DNA also was detected in a subset of cells within dysplastic regions. Although a number of cells in dysplastic regions exhibited very strong staining for HPV-16 E6/E7 DNA, staining was not detected in a number of neighboring cells in the dysplastic region. In contrast, throughout the CIN II biopsies examined HPV-16 E6/E7 mRNA was not typically detected in basal cells, proliferating squamous cell precursors, columnar epithelium of the endocervix, or underlying stromal cells, and was not necessarily detected in regions of epithelial dysplasia.

Detection of HPV-16 E6/E7 DNA in Epithelia with Abnormal Squamous Cell Maturation

To determine if the distribution of HPV-16 E6/E7 DNA was associated with regions exhibiting histopathic changes associated with viral infection or neoplasia, 50

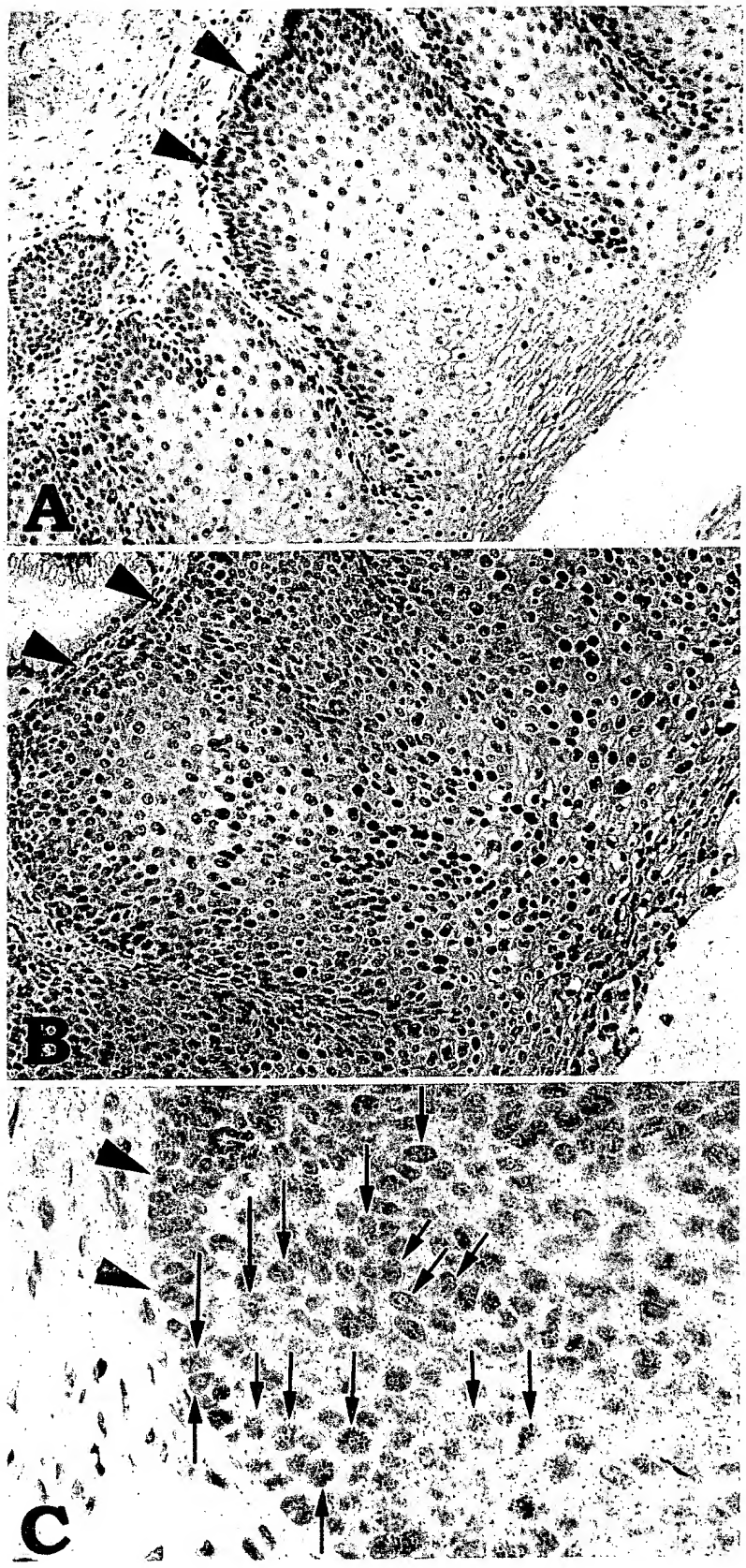

Figure 2 Detection of HPV-16 E6/E7 DNA in epithelial regions with abnormal squamous cell maturation. The presence of viral sequences is visualized by deposition of the Fast Red–AP reaction product (red). For visualization of cell nuclei, sections were counterstained with hematoxylin (blue). **(A)** HPV-16 E6/E7 DNA is not detected in a region of epithelium that exhibits normal squamous cell maturation. **(B)** HPV-16 E6/E7 DNA is detected in adjacent regions of the same tissue section that exhibit highly abnormal squamous cell maturation in CIN II specimen 624. **(C)** High-magnification view of epithelial regions with abnormal squamous cell maturation shows that HPV-16 E6/E7 DNA is detected in nuclei of basal cells and parabasal cells near the basement membrane, as indicated by arrows. Arrowheads mark location of basement membrane. Original magnifications: **A,B** ×400; **C** ×600.

sections of each of the HPV-positive biopsies were extensively examined using bDNA ISH. Figures 2A and 2B show two adjacent epithelial regions from the same tissue section of specimen 624. Figure 2A shows a region of normal squamous cell maturation in which HPV-16 E6/E7 DNA was not detected. By contrast, Figure 2B shows a nearby region of the same epithelium that exhibited highly abnormal squamous cell maturation, with moderate dysplasia marked by overgrowth of basal cells and mixing of undifferentiated cells, differentiated squamous cells, koilocytes, and dysplastic cells. In this region, HPV-16 E6/E7 DNA was detected in nuclei of cells exhibiting koilocytic changes and in some cells close to the basement membrane. However, similar to specimen 627, HPV-16 E6/E7 DNA was predominantly detected in regions of cells with koilocytic changes and was not necessarily detected in areas of dysplasia.

Detection of HPV-16 E6/E7 DNA in Basal Cells of CIN II Lesions

With the bDNA ISH method it was also possible to identify individual basal cells that contained HPV-16 E6/E7 DNA. As shown in Figure 2C, the relatively weak signal for HPV-16 E6/E7 DNA detection in basal cells and parabasal cells near the basement membrane was more clearly distinguished from background by incorporating an antigen-unmasking pretreatment step in the bDNA ISH protocol. Although HPV-16 E6/E7 DNA was detected in some basal cells throughout specimen 624, it was not detected in basal cells of other CIN II specimens examined. In addition to HPV-16 E6/E7 DNA detection in basal cells, specimen 624 also exhibited a greater number of HPV-infected cells and a greater preponderance of signal in these cells.

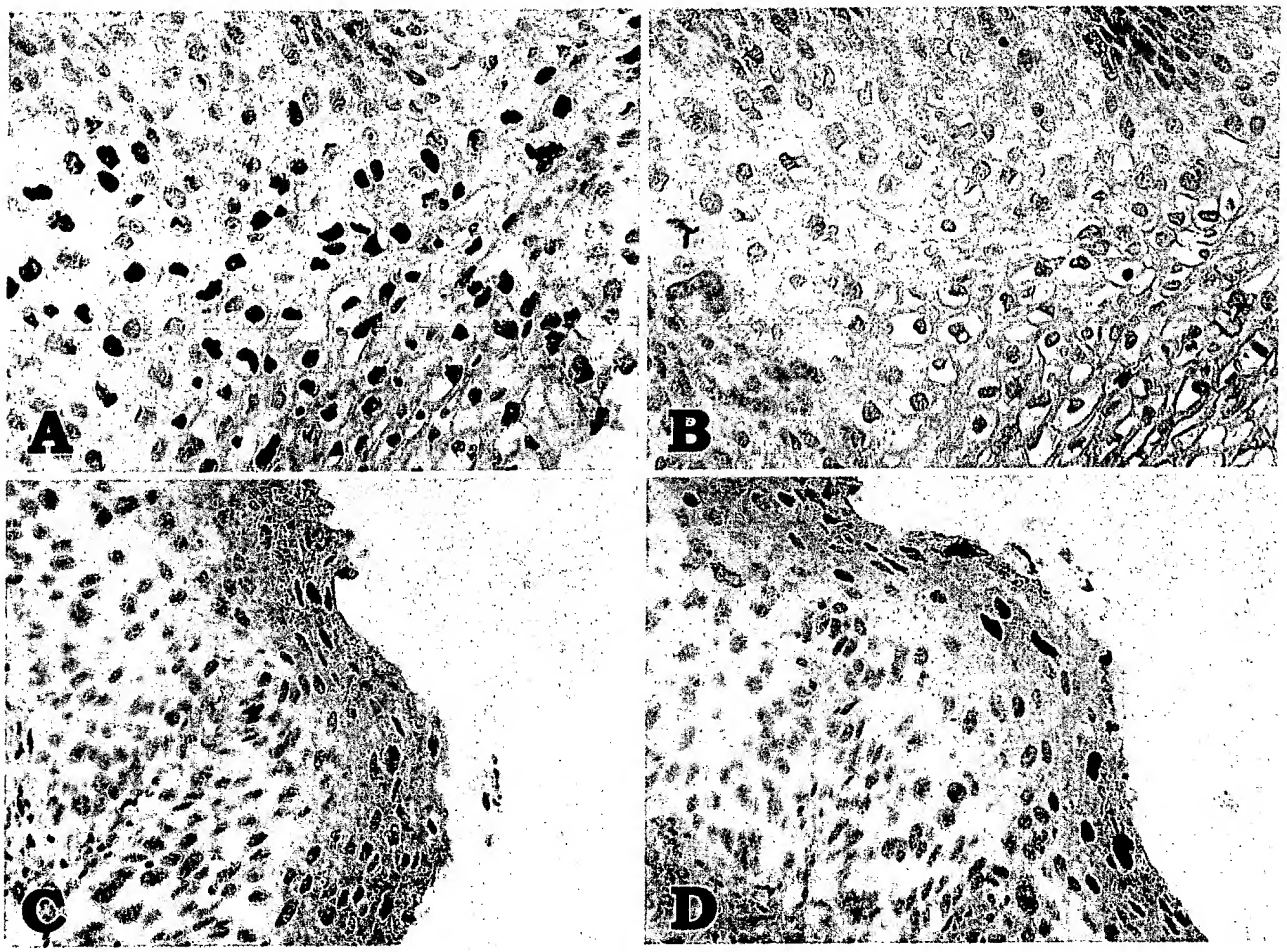

Figure 3 Genotype-specific detection of HPV E6/E7 DNA in CIN II lesions. The presence of viral sequences is visualized by deposition of the Fast Red alkaline phosphatase reaction product (red). For visualization of cell nuclei, sections were counterstained with hematoxylin (blue). (A) Hybridization of HPV-16 E6/E7 probes to DNA yields signal localized predominantly to nuclei of koilocytic cells in CIN II specimen 624. (B) HPV-18 E6/E7 probes do not hybridize to CIN II specimen 624. (C) HPV-16 E6/E7 probes do not hybridize to CIN II specimen 625. (D) Hybridization of HPV-18 E6/E7 probes indicates presence of HPV-18 E6/E7 DNA in CIN II specimen 625. Original magnification ×600.

Genotype-specific Detection of HPV-16 and HPV-18 E6/E7 DNA in CIN II Lesions

Tissue sections from multiple CIN II biopsies were analyzed to compare the distribution of HPV-16 and HPV-18 E6/E7 DNA. Figure 3 shows sections from two CIN II cervical tissues that were analyzed with bDNA ISH. HPV-16 E6/E7 DNA was detected in CIN II specimen 624 (Figure 3A) but not in CIN II specimen 625 (Figure 3C). By comparison, HPV-18 E6/E7 DNA was detected in CIN II specimen 625 (Figure 3D) but not in CIN II specimen 624 (Figure 3B). These results demonstrate that the detection of HPV DNA in CIN II lesions using bDNA ISH was genotype-specific.

Discussion

In our previous study we demonstrated single-copy gene detection sensitivity for bDNA ISH using cell lines (Player et al. 2001). The present study demonstrates the efficacy of bDNA ISH for detecting nucleic acid sequences in clinical tissue specimens. Our results show that bDNA ISH yielded rapid, sensitive detection of HPV-16 or HPV-18 E6/E7 DNA and mRNA with high specificity while preserving cervical tissue morphology. The specificity of probe hybridization in CIN II biopsies was sufficient to identify biopsy specimens infected with HPV and to distinguish among different HPV genotypes. Furthermore, cell morphology was preserved well enough to distinguish certain cell types that were infected by HPV from neighboring cells in which HPV was not detected. Even in tightly packed heterogeneous epithelia, individual koilocytes that expressed HPV-16 E6/E7 mRNA were clearly distinguished from neighboring cells that were negative for HPV-16 E6/E7 mRNA. Although in cell lines bDNA ISH has been shown to detect as few as one or two copies of DNA per cell (Player et al. 2001), our study did not directly assess the sensitivity of bDNA ISH in tissue sections and it is difficult to make direct comparisons between cell lines and tissues. However, our observation that HPV-16 E6/E7 DNA was detected in individual basal cells, which are thought to have low copy numbers of HPV (Stoler and Broker 1986), indicates that the sensitivity of bDNA ISH in tissues is sufficient to detect low-abundance molecules.

In overcoming the challenges for localization of nucleic acid sequences in clinical tissue sections, bDNA ISH offers many advantages over target amplification methods such as PCR ISH. For example, the histopathological features of formalin-fixed, paraffin-embedded tissue sections are well preserved with bDNA ISH, in part because bDNA signal amplification does not require repeated heat cycling (Nuovo et al. 1991). Because bDNA ISH does not need polymerase activity, it is not affected by the unpredictable presence of polymerase inhibitors in tissue specimens that may affect reproducibility of PCR ISH methods (Daniel et al. 2000). Moreover, because the AP-conjugated reporter bDNA probe is physically linked to the target nucleic acid, the reaction product is retained at the site, thus allowing precise subcellular localization of the target nucleic acid and avoiding the problems of diffusion and false-positives associated with PCR ISH methods (Nuovo et al. 1991; Wiedorn et al. 1999).

Differences in technology distinguish bDNA from other signal amplification systems such as tyramide signal amplification (TSA) applied to ISH. TSA and bDNA ISH have similar sensitivities in cell lines and are effective in detection of target sequences in tissues (Kerstens et al. 1995; Plummer et al. 1998; Player et al. 2001). Although both bDNA and TSA improve the sensitivity of ISH by increasing the numbers of binding sites for reporter molecules, they use different mechanisms to achieve amplification of the signal. TSA is based on the application of biotinylated tyramide peroxidase substrate to the avidin–biotin system and therefore relies on the high affinity constant and specificity of the avidin–biotin complex (Bayer and Wilchek 1978; Wilchek and Bayer 1988). In contrast, bDNA is based on hybridization of nucleic acids and therefore relies on stringent hybridization conditions involving complementary sequences of oligonucleotides, elevated temperatures, the addition of non-natural nucleotides that reduce nonspecific binding, reduced probe concentration, and stringent washing conditions involving buffers with decreasing salt concentrations. Because bDNA signal amplification is based on the hybridization of deoxyoligonucleotides rather than on the protein–protein interactions of TSA, it affords a number of operational opportunities to control the signal amplification specificity not available to the TSA system. The two methods also differ in the type of reporter system used. TSA ISH uses peroxidase-dependent tyramide technology that is either fluorescent or chromogenic (van de Corput et al. 1998), whereas bDNA ISH uses an AP-catalyzed reporter system that provides the opportunity to use AP substrates such as the highly sensitive Fast Red chromogen substrate (DAKO), that is both chromogenic and fluorescent (Player et al. 2001).

Although this study focused primarily on the efficacy of bDNA ISH for detection of HPV in cervical tissue specimens, bDNA ISH may be applicable to genotype- and cell type-specific detection of high-risk HPV genotypes in other tissues with HPV-associated cancers, such as head and neck (Gillison et al. 2000; Mork et al. 2001), lung (Cheng et al. 2001), and melanoma (Dreau et al. 2000). In addition, because oligonucleotide probes for bDNA signal amplification can be readily designed (Bushnell et al. 1999), bDNA ISH can be developed for detection of other nucleic acid

sequences that are related to cancer and infectious disease. For example, we have reproducibly detected HIV-1 RNA in lymph node tissue from HIV-1-infected asymptomatic individuals (data not shown). These results suggest that bDNA ISH may provide a specific and sensitive means for detecting genetic markers and gene expression in a variety of specimen types.

Studies using sensitive techniques such as bDNA ISH to further investigate the topographical distribution of HPV and neoplasia in cervical biopsies might offer unique insight into the disease process. Although the potential clinical role of HPV testing is controversial (Kaufman and Adam 1999; Manos et al. 1999; Bishop et al. 2000; Bovicelli et al. 2000), infection with high-risk HPVs has been associated with the progression of intraepithelial precursor lesions to cervical carcinoma (zur Hausen 1996), and HPV-16 and HPV-18 have consistently been found in over 90% of cervical carcinomas and in 50–80% of CIN lesions (Arends et al. 1998). In this study we found that HPV-16 E6/E7 mRNA and DNA were present in areas of cells with koilocytic changes but not necessarily in regions of dysplasia. Our observations suggest that HPV E6/E7 gene expression may not be directly associated with the overgrowth of epithelial progenitors and dysplastic cells but rather may influence neoplastic processes indirectly. However, examination of a greater number of CIN biopsies as part of a comparative clinical study would be needed to determine the association between the distribution of E6/E7 mRNA and DNA from high-risk HPV genotypes and the development of high-grade squamous intraepithelial lesions.

In summary, bDNA ISH is a reproducible, sensitive, specific, and precise method for localizing nucleic acid sequences in clinical tissue sections with exquisite preservation of tissue morphology. As an adjunct to conventional histopathology, bDNA ISH may contribute to the understanding, diagnosis, and prognosis of disease.

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